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13. ABSTRACT *(Maximum 200 Words)*

The overall goal of our research is to investigate the correlation between BRCA1-mutated breast cancers and the Estrogen Receptor (ER)-negative phenotype. During the past year, we have focused on analysis of the CpG methylation status of the ER promoter, a proposed molecular mechanism by which sporadic ER-negative tumors lack expression of ER. We have analyzed a panel of human breast cancer cell lines and a collection of specimens from ER-negative breast cancers by two established assays. The one cell line in our panel that derived from a BRCA1 mutation carrier (HCC1937) was significantly hypomethylated, relative to the other ER-negative lines. In contrast, DNA from ER-negative BRCA1-linked breast cancers was somewhat more methylated than a parallel cohort of ER-negative non BRCA1-linked cancers, indicating that the HCC1937 line is not representative of BRCA1-derived tumors in this regard. We have recently developed a semi-quantitative sequence analysis-based method to assay a greater number of CpG sites within the ER promoter, and will apply this to our specimens.

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Overview:

The overall goal of our research is to investigate the correlation between BRCA1-mutated breast cancers and the Estrogen Receptor (ER)-negative phenotype. Although most sporadic breast cancers are ER-positive, studies have consistently shown that the vast majority of BRCA1-associated breast cancers are ER-negative (1-3). In sporadic cancers lacking ER expression, decreased expression of ER mRNA has been noted, without genomic DNA mutations in the ER gene (4-8). Methylation of CpGs within the ER promoter has been implicated as an operative mechanism of repressed expression in some cell lines and tumor specimens. In accordance with the Statement of Work, the majority of effort in this second year of funding was devoted to our first aim: to compare the methylation status of selected CpG sites located in the ER promoter region between ER-negative BRCA1-associated breast cancers and BRCA1-wildtype ER-negative tumors. We have also begun to prepare the necessary plasmids to conduct the experiments outlined for the third year of funding.

Progress to Date:

In last year's annual report, we detailed our work developing and standardizing assays of CpG methylation at the ER promoter that we planned to apply to a collection of ER-negative breast cancers. We can now report our results from the analysis of patient specimens by two assays. First, we used the so-called Methylation Specific PCR (MSP) assay of bisulfite-treated DNA, based on the use of PCR primers which selectively target selected clusters of CpGs, taking advantage of the fact that the pre-PCR bisulfite treatment will change unmethylated CpGs into TpGs, while leaving methylated CpGs unaltered. For this assay, we selected primer pair called 'ER1' by Davidson's group (9). As a complementary assay, one not dependent upon bisulfite treatment and modification of the DNA, we applied the assay of Iwase *et al.* in which genomic DNA was first digested with the methylation-sensitive enzyme HpaII, then amplified with primers that span the restriction cut site (10).

We compared 18 ER-negative breast cancers from women with documented germline mutations in BRCA1 to a collection of 18 ER-negative breast cancers not linked to BRCA1. Specimens came from our own institution, as well as from collaborators at Memorial Sloan Kettering, Cleveland Clinic, and University Hospital in Lund, Sweden. As shown in the column labeled 'HpaII Digest' in Table I, significant methylation was evident in the BRCA1-linked specimens (94%). Among the non BRCA1-linked group 81% showed methylation, consistent with published data (10). While the difference between the BRCA1-linked and non BRCA1-linked groups was not statistically significant ($p > 0.2$), these data suggest that our findings with the HCC1937 cell line (reported in last year's report) are not representative of primary BRCA1-linked breast cancers.

Table I also contains data from MSP analysis of DNA from the patient specimens, using the ER1 primers. As a semi-quantitative measure of the relative abundance of methylated and unmethylated DNA at the ER1 primer binding sites, we amplified bisulfite-treated DNA with the methylated DNA-specific primer pair as well as with the degenerate primer pair in parallel amplification reactions and compared the relative intensity of the resulting PCR products on an agarose gel. In separate reactions utilizing synthesized templates representing methylated and unmethylated ER1 sequence, we determined that PCR products of equal intensity with these primer pairs resulted when the methylated DNA template constituted ~10% of the total, probably reflecting a lower efficiency of amplification with the

degenerate primers (not shown). We observed that whereas only 1 out of 12 samples in the non BRCA1-linked group produced a PCR band of greater intensity with the methylated DNA-specific primers than with the degenerate primers, half of the BRCA1-linked group (4 out of 8) yielded a PCR band of greater intensity with the methylated DNA-specific primers than with the degenerate primers. This represents a significant difference between the two groups ($p = 0.035$ by Chi-Square analysis), consistent with a higher level of CpG methylation being present in the cancers from BRCA1 mutation carriers.

The data in Table I indicate that the hypothesis that BRCA1-linked breast cancers will be notably unmethylated, as was the case with the HCC1937 cell line derived from a BRCA1 mutation carrier, is probably incorrect. Indeed, the MSP data with the ER1 primer pair suggest the opposite hypothesis. A significant limitation of these data, however, is that they reflect methylation status at only a few CpG sites (those within the HpaII restriction sites flanked by the specific PCR primers, and those within the MSP primer sequences).

Table I. Evaluation of CpG methylation in ERa-negative breast cancers by *HpaII* digest and MSP analysis

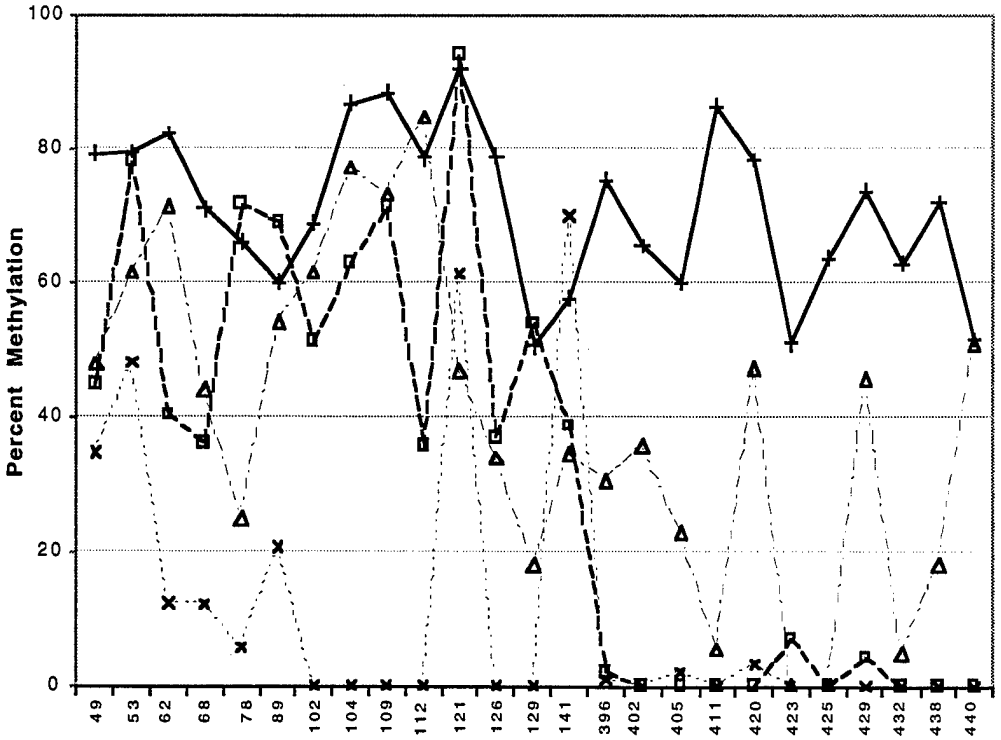
Non *BRCA1* linked	*HpaII* Digest[a]	MSP[b]	*BRCA1* linked	HpaII *Digest*	MSP
S1	m	↓	B1	m	↑
S2	m	=	B2	m	NA
S3	m	↓	B3	m	=
S4	m	=	B4	m	=
S5	m	↓	B5	u	NA
S6	u	=	B6	m	↑
S7	m	↓	B7	m	NA
S8	m	↓	B8	m	↓
S9	m	NA	B9	m	NA
S10	NA	↓	B10	m	NA
S11	m	↓	B11	m	↑
S12	u	↑	B12	m	↑
S13	NA	↓	B13	m	↓
S14	u	NA	B14	m	NA
S15	m	NA	B15	m	NA
S16	m	NA	B16	m	NA
S17	m	NA	B17	m	NA
S18	m	NA	B18	m	NA

[a] Notations include the following: m, methylated; u, unmethylated; NA, no DNA detected.

[b] Bisulfite-treated DNA was amplified with the ER1 methylated DNA-specific primers as well as with ER1 degenerate primers, then visualized by agarose gel, as described (9). Indicated is whether the intensity of the PCR product with the methylated DNA-specific primers was greater than (↑), less than (↓), or equal to (=) the intensity of the PCR product obtained with the degenerate primers. NA, no amplification with either primer pair.

We are now proceeding with examination of methylation status by sequence analysis of bisulfite-treated DNA. To develop and standardize the methodology, we first analyzed DNA from a panel of four ER-negative human breast cancer cell lines. The ER1 and ER5 regions were amplified from bisulfite-treated DNA with degenerate primers, such that both methylated and unmethylated template DNA would be co-amplified. The resulting PCR products were then directly sequenced, thereby providing data on 25 CpGs located within the amplified regions. Conversion of the non CpG cytosines was >95%, indicating that incomplete bisulfite treatment was not the reason for heterogeneity of methylation noted in the MSP analysis. Figure 1 shows the percent methylation at each CpG site in our panel of ERα-negative cell lines. In support of the MSP data reported in last year's report, MCF10A cells showed the highest level of methylation while HCC1937 cells showed the lowest level of methylation across the ER1 and ER5 regions. MDA-MB-231 cells, showing a heterogeneous MSP signal, were confirmed by sequencing to have an overall percent methylation between that of MCF10A cells and HCC1937 cells. 184B5 cells were shown by sequencing, as with MSP, to be highly methylated in ER1, but largely unmethylated in ER5. As a measure of methylation across the ER1 and ER5 sequenced regions, we averaged the percent methylation of all CpG sites. The average percent methylation was 71% in MCF 10A cells, 11% in HCC1937 cells, 39% in MDA-MB-231 cells and 32% in 184B5 cells. Of note, only the 184B5 cells demonstrated markedly different degrees of methylation between the ER1 and ER5 regions (56% in ER1 vs. 1% in ER5). We are now applying this methodology to DNA from patient specimens.

Figure 1. CpG methylation of the *ERα* promoter in four ERα-negative cell lines. Bisulfite-treated DNA from each cell line was amplified with degenerate primers for the ER1 and ER5 regions, and then sequenced. CpG location is indicated (*x axis*) relative to the first nucleotide of P1. MCF10A cells (+ connected with a *solid black line*), MDA-MB-231 cells (Δ connected with a *dashed black line*), 184B5 cells (□ connected with a *dash-dot gray line*) and HCC1937 cells (x connected with a *dotted gray line*) are shown.



The third year of funding will largely be devoted to a test of the hypothesis that BRCA1 exhibits transcriptional activation activity towards the ER promoter. To prepare for these experiments, we have prepared an ER promoter construct driving expression of luciferase. First, we obtained as a generous gift a construct from Dr. R.J. Weigel in which ~3.5kb of sequence upstream of the transcription initiation site of the main (P1) promoter and 210 bp of downstream 5' untranslated sequence from the ER promoter was linked with luciferase in the pGL2 vector (11). The work of Tang *et al.* have provided evidence suggested the presence of an enhancer element a bit upstream of this sequence, which they called ER-EH0 (12). We used PCR amplification from genomic DNA to amplify and then subclone into the promoter construct obtained from Dr. Weigel an additional 310 bp of upstream sequence such that our ER promoter-luciferase construct now incorporates all known enhancer elements. This construct, and 5' deletions to be derived from it, will be used to pursue our second aim in the coming year.

Key Accomplishments

- Specimens or sporadic and BRCA1-derived ER-negative breast cancers have been analyzed at selected CpG sites within the ER-promoter, suggesting an increased level of methylation within the BRCA1-linked tumors.
- Methodology for sequence-based analysis of methylation at 25 CpG within the ER promoter, following bisulfite modification of genomic DNA, has been developed and standardized with a panel of human breast cancer cell lines.
- An expanded ER promoter-luciferase construct, incorporating all known transcriptional enhancer elements for ER expression, has been constructed and will be used in the coming year to test the hypothesis that BRCA1 transactivates the ER promoter.

Reportable Outcomes

none

Training Activities

In this past year, the supported student, having completed all course requirements, has focused full-time effort on the research.

Conclusions

We had initially hypothesized that ER-negative breast cancers from BRCA1 germline mutation carriers would be notably less methylated than ER-negative non BRCA1-linked tumors. In part, this tentative hypothesis derived from our prior results showing that the HCC1937 cell line (an ER-negative line from a BRCA1 mutation carrier) demonstrated significantly less methylation than sporadic ER-negative cell lines. Our data thus far indicate that the opposite is true: ER-negative BRCA1-linked breast cancers are more highly methylated than ER-negative non BRCA1-linked tumors. To clarify and quantitate this further, we have perfected the approach of sequence-based analysis of bisulfite-treated DNA and will analyze our collections of DNA from patient specimens by this approach.

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